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14. ABSTRACT

A summary is presented of research performed during the first year of a project to determine feasibility of approaches to restore wild-type transcriptional activity on mutant p53 proteins found in human prostate tumors. p53 mutant proteins that are specifically relevant to prostate cancer are being examined to determine whether they are suitable targets for such an approach. Three specific aims are being pursued. The first is characterizing the interaction of p53 with two distinct classes of its response elements. The second aim is determining the role of mutant p53 proteins in prostate cancer cell proliferation. The final aim is to explore approaches to restore wild-type function to mutant p53 proteins found in prostate cancer. The long term goals of this research is to identify small molecular weight compounds which have the novel activity of restoring wild-type function to prostate cancerderived mutant p53 proteins. As such, this represents a preclinical development of highly targeted therapy with the hope of establishing highly effective and tumor-specific treatments of human prostate cancer.

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p53, DNA binding, transcription, gene expression

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Introduction

Our laboratory has demonstrated the existence of two distinct classes of p53 binding sites (Resnick-Silverman et al., 1998; Thornborrow and Manfredi, 1999; St. Clair et al., 2004). Thus, studies are underway to characterize these distinct types of p53 elements and to utilize both in screens for ways to restore wild-type p53 activity in prostate cancer cells expressing mutant p53. Three specific aims are being pursued. The first is characterizing the interaction of p53 with two distinct classes of its response elements. The focus of this aim is a full understanding of the nature of these two subsets of p53 response elements and elucidation of the molecular details of the interaction of p53 with different kinds of binding sites. Such knowledge will be critical for pursuing approaches that restore activity to mutant p53 proteins. The second aim is determining the role of mutant p53 proteins in prostate cancer cell proliferation. Approaches to abrogate mutant p53 expression and to express mutant p53 proteins in both tumorigenic and normal prostate cells are being used to determine the role of mutant p53 protein in cell proliferation. "Proof-of-principle" experiments will be performed to show the feasibility and usefulness of restoring wild-type activity in mutant p53-expressing prostate tumor cells. The final aim is to explore approaches to restore wild-type function to mutant p53 proteins found in prostate cancer. As the specific mutations found in prostate cancer are distinct from the well-studied "hot spot" mutants, as part of this aim, the activity of these various mutant p53 proteins are being examined. In vitro DNA binding assays are determining the temperature-sensitivity of these various mutant p53 proteins. The ability of previously identified methods for re-activating DNA binding in mutant p53 is being tested for these mutants. Finally, a cell-based reporter assay system is being established to allow for rapid screening of compounds that can restore wild-type transcriptional activity on prostate tumor-derived mutant p53 proteins.

Body

Task 1. Validate the existence of different classes of p53 response elements (Months 1-12)

Seventeen different p53 response elements were placed upstream of a heterologous promoter (that of the minimal adenovirus E1B) driving luciferase expression and were compared in transient transfection assays. In parallel, the same DNA sequences were used in electrophoretic mobility shift assays to compare the relative binding affinity for p53. Although there was a general correlation between in vitro DNA binding and transcriptional activation in the luciferase assay, a subset of sites showed robust binding by p53 in vitro but only minimal activation in the cell-based assay. Two of these elements, the downstream site from the p21 promoter (p21 3') and the upstream site from the PIG3 promoter (PIG3 5') were examined more closely in comparison to two other sites, that from the 14-3-3sigma gene and from the cyclin G1 promoter. These results are shown in Figure 1. While the p21 3' and the 14-3-3sigma sites bound to p53 in a similar manner in vitro, but the 14-3-3sigma site conferred robust p53-dependent transcriptional activation whereas the p21 3' site did not. Similarly, the cyclin G and PIG3 sites showed comparable binding to p53, but only the cyclin G site behaved as a strong p53 response element in this assay. These results validate the existence of two classes of p53 sites which are bound in a comparable manner to p53 but differ in their ability to act as response elements. Previous studies have shown that a monoclonal antibody which binds at the C-terminus of p53 enhances binding to one set of sites and inhibits binding to the other set. It had been previously suggested that the binding of the antibody mimics post-translational modification of p53 at the C-terminus, notable acetylation. Similar results have been obtained by examining mutant p53 proteins which have substitutions at lysines in the C-terminus which have been implicated as sites of acetylation. Mutation to arginine (conserving charge) does not confer differential binding to the two classes of sites whereas substitution with glutamine (which has been suggested to mimic constitutive acetylation) does cause the mutant p53 to bind with distinct affinity to the different sites (Figure 2). Finally, the ability of p53 to bind to oligonucleotides of varying lengths was examined in in vitro DNA binding assays. The binding of p53 to the p21 5' site appears to depend upon the length of the oligonucleotide in an electrophoretic mobility shift assay, whereas the p21 3' site binding is unaffected (Figure 3). Thus, multiple assays suggest that p53 can interact with different classes of p53 sites in a distinct manner, most likely by adopting a different conformation that is determined by the DNA sequence. Studies are now underway to explore this hypothesis in more detail as outlined in the research plan.
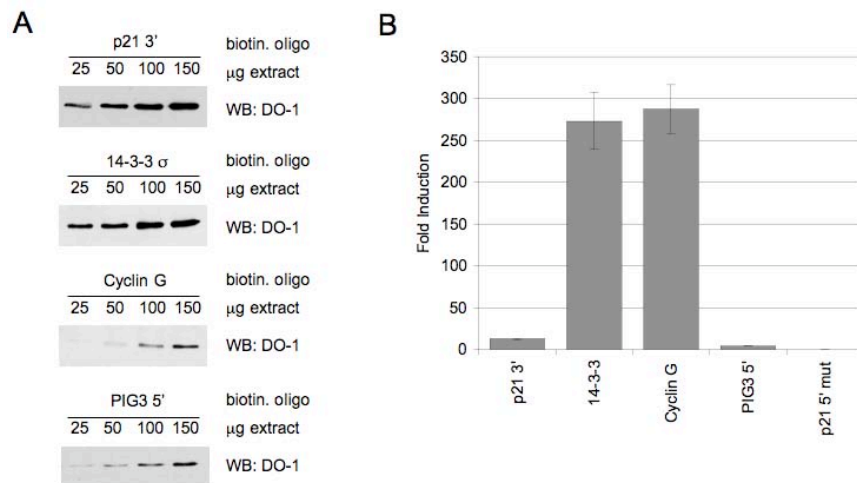
Figure 1: DNA affinity of p53 cells does not correlate with transcriptional activation for a subset of response elements. (A) 25, 50, 100 and 150 mg of extracts were incubated with the indicated biotinylated oligonucleotides. DNA-protein complexes were precipitated with streptavidin beads and the presence of p53 was assayed by Western blot. (B) Cells were transfected with 1 mg of the indicated luciferase reporter constructs and 50 ng of pRL-Renilla. 24 hrs post transfections p53 was induced by removal of tetracyline. Cells were lysed and assayed for luciferase and Renilla activities 24 hrs after induction of p53. The indicated values are the average of three independent experiments, each performed in duplicates.
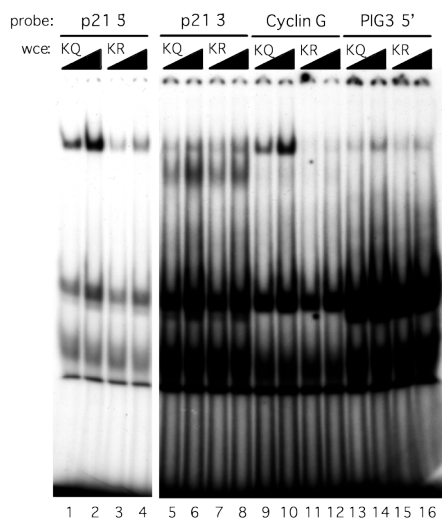

Figure 2. C-terminal mutation affects binding of p53 to a subset of p53 response elements. EMSAs were performed incubating probes containing the p21 5' (lanes 1-4), p21 3' (lanes 5-8), cyclin G (lanes 9-12) or PIG3 5' (lanes 13-16) p53 response elements with two levels of extracts from cells transfected with pCMV-p53^{KQ} or –p53^{KR} mutants.
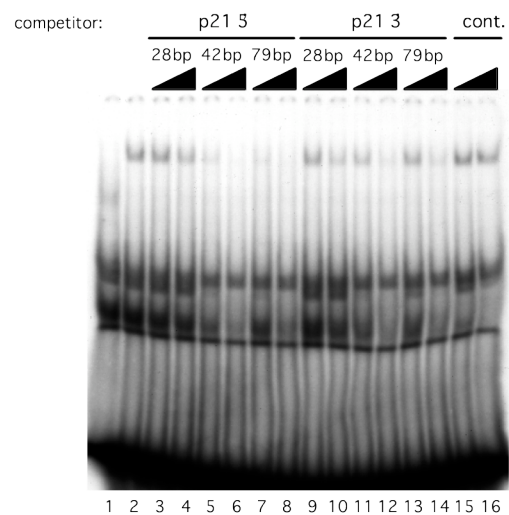

Figure 3. Length of DNA oligonucleotide influences binding of p53 to a subset of response elements. EMSAs were performed incubating the 42-bp radiolabeled p21 5' oligonucleotide with extract from p53-transfected H1299 cells in the presence of mAb 1801 (lanes 2-16). Binding of p53 to the probe was competed with 30- (lanes 3, 5, 7, 9, 11, 13 and 15) or 120-fold (lanes 4, 6, 8, 10, 12, 14 and 16) molar excess of different length unlabeled oligonucleotides containing the p21 5' or 3' response elements as indicated.

Task 2. Determine whether p53 adopts distinct conformations on subsets of its response elements (Months 6-15)

Conditions were established for limited chymotrypsin proteolysis of purified p53 protein in the presence of radiolabeled oligonucleotides containing either the p21 5' site or the p21 3' site. These mixtures were then subjected to electrophoretic mobility shift analysis. Limited proteolytic digestion lead to enhanced binding of p53 to the p21 5' site whereas in the presence of the p21 3' site binding was substantially reduced (Figure 4). However, the effects that were seen were similar whether the DNA was present during the proteolysis or added after the proteolysis reactions were terminated. This indicates that this approach will not be useful for determining whether p53 adopts distinct conformations on the different sites. Nevertheless, these results

suggest that limited proteolysis of p53 generates fragments with differing affinity for subsets of binding sites. Further analyses demonstrated that indeed the fragment that shows enhanced binding to the p21 5' site and decreased binding to the p21 3' site lacks the C-terminus. This finding was validated with several other p53 sites representing each class of response element. Use of a mutant p53 which lacks the last 30 amino acids confirmed that similar results can be obtained. Thus, whether the C-terminus is removed by limited proteolysis of through genetic means, the findings were similar and confirm the results obtained with the monoclonal antibody and C-terminal lysine mutants as described above. It remains an important goal to rigorously address the possibility of conformational differences of p53 when bound to different sites. With this in mind, studies are currently underway to explore crosslinking of the p53 to the DNA prior to digestion as a means to achieve this. Thus, these studies will continue into the next funding year.
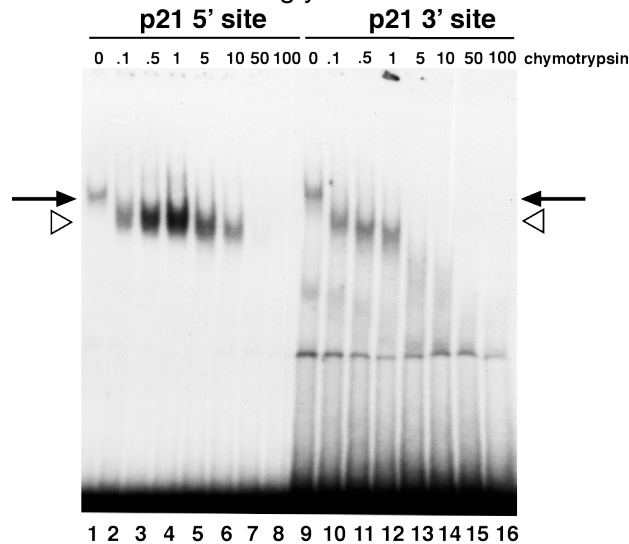

Figure 4. Digestion with chymotrypsin produces truncated forms of p53 which have selectively enhanced binding to the p21 5' site as compared to the p21 3' site. Electrophoretic mobility shift assays were performed using oligonucleotides corresponding to either the p21 5' site or p21 3' site as radiolabeled probe. 10 ng of purified p53 was digested with increasing amounts of chymotrypsin (0-100 ng) for 20 minutes and EMSA was performed in the presence of 500 ng of non-specific poly (dI/dC) competitor DNA.

Task 3. Determine the significance of distinct classes of p53 response elements (Months 16-27)
This task is to be performed in a future funding year.

Task 4. Determine the role of p53 in mutant p53-expressing DU145 prostate cancer cells (Months 1-27)
An siRNA approach to ablate expression of mutant p53 in the DU145 cell line will be used to determine whether mutant p53 expression is necessary for DU145 cell proliferation. Although it was proposed to use a stable shRNA method, as these studies were being performed, the concern arose that if mutant p53 is required for proliferation, it may be difficult to determine this using stable transfection. Thus, conditions for transient downregulation of mutant p53 using transfection with siRNA oligonucleotides were explored. The high level of mutant p53 expression in DU145 cells as made this a more difficult task than anticipated. Although methods to ablate p53 in wild-type p53 expression have been quite successful in the laboratory, the level of mutant p53 in DU145 is approximately 10-fold higher than that seen in the wild-type p53 expressing cells. The original approach had been to use a single siRNA oligonucleotide. Although this worked well in the wild-type p53 cells, it was ineffective in DU145 cells. Thus, it was determined that a mixture of four different oligonucleotides was needed to successful downregulate mutant p53 expression in DU145 cells by greater than 95%. With this method in hand, studies are now underway to determine using flow cytometric approaches whether loss of mutant p53 expression in DU145 cells affects the cell cycle profile as well as the ability to incorporate bromodeoxyuridine (as a reflection of DNA synthesis rate). Based on the results from these studies, it will be determined whether stable transfection approaches are warranted.

Task 5. Examine the role of prostate tumor-derived mutant p53 proteins in regulating cell proliferation (Months 15-31)
This task is to be performed in a future funding year.

Characterize previously identified methods to restore wild-type function on prostate tumor-derived mutant p53 proteins (Months 24-36)
This task is to be performed in a future funding year.

Task 7. Establish approaches to screen for compounds which restore the ability of prostate tumor-derived mutant p53 proteins to activate transcription (Months 1-36)
Four p21 reporter constructs were generated as shown in Figure 5. These included the full-length p21 promoter, one that lacks the upstream 5' site, one that lacks the downstream 3' site, and a construct which lacks both sites. The activity of these reporters was confirmed in transient transfection assays as shown in Figure 5. The luciferase cDNA was then replaced with a cDNA encoding green fluorescent protein (GFP) and the activity of these reporters were then examined in similar transient transfection assays. It was confirmed that GFP expression was obtained in response to co-transfection with wild-type p53 and that each of the mutant reporters showed less activation with the construct lacking both sites showing minimal GFP expression. Studies are now underway to make stable cell lines expressing each of these reporters and to characterize copy number and activity as the next step in establishing a high-through put approach to screen compounds for the ability to restore wild-type activity to mutant p53 proteins.
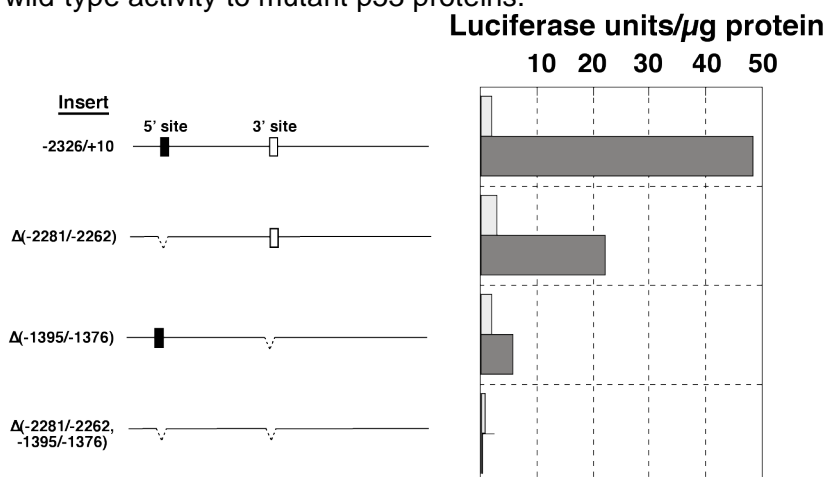

Figure 5. Both the 5' and 3' sites are required for p53-dependent transcriptional activation. The indicated luciferase reporters were generated and co-transfected into p53-null cells with an expression plasmid expressing wild-type p53. The average of three independent experiments performed in duplicate is shown.

Key Research Accomplishments

- Maurer, M., Resnick-Silverman, L., Thornborrow, E., and Manfredi, J.J.. The C-terminus of p53 selectively regulates binding to a distinct subset of response elements: support for an allosteric model for regulation of DNA binding. In preparation.
- Giono, L.E., Resnick-Silverman, L., and Manfredi, J.J. p53 adopts different conformations on subsets of its response elements. In preparation.

Reportable Outcomes

- Generated luciferase reporters containing 17 different p53 response element
- Generated expression plasmids for C-terminal p53 mutants as well as two prostate-specific tumor derived p53 mutants
- Generated reporters which express green fluorescent protein (GFP) under control of the p21 promoter as well as p21 promoters which lack either the upstream p53 site (p21 5'), the downstream p53 site (p21 3') or both

Conclusions

The long term goals of this research is to identify small molecular weight compounds which have the novel activity of restoring wild-type function to prostate cancer-derived mutant p53 proteins. As such, this represents a preclinical development of highly targeted therapy with the hope of establishing highly effective and tumor-specific treatments of human prostate cancer. Much of the specific research being performed is

laboratory-based and focused on feasibility of such an approach. Nevertheless, it represents necessary preliminary studies which will allow further development and translation of these findings in the future with the ultimate goal of establishing a highly effective and targeted therapy for human prostate carcinoma.

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Thornborrow, E. C., and J. J. Manfredi. 1999. One mechanism for cell type-specific regulation of the bax promoter by the tumor suppressor p53 is dictated by the p53 response element. J Biol Chem 274:33747-56.

St Clair, S., Giono, L., Varmeh-Ziaie, S., Resnick-Silverman, L., Liu, W.J., Padi, A., Dastidar, J., DaCosta, A., Mattia, M., and Manfredi, J.J. 2004. DNA damage-induced downregulation of Cdc25C is mediated by p53 via two independent mechanisms: one involves direct binding to the cdc25C promoter. Mol Cell 16:725-736.

Appendices
None.